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OBSERVATIONS ON HEPARIN AND HEPARIN ANTITHROMBIC COFACTOR*

by

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ABSTRACT

OBSERVATIONS ON HEPARIN AND HEPARIN ANTITHROMBIC COFACTOR

OBJECT

To study the action of heparin and its antithrombic cofactor on the *in vitro* conversion of fibrinogen to fibrin by thrombin.

RESULTS AND CONCLUSIONS

Heparin exerts an immediate, moderate, and reversible antithrombic action detectable only at low concentrations of thrombin. The addition of heparin antithrombic cofactor to a mixture of thrombin and heparin produces an immediate, irreversible inactivation of thrombin in proportion to the amount of cofactor added. The concentrations of thrombin, heparin, and cofactor, respectively, were found to be critical in determining the individual contribution of heparin and of the cofactor to their combined antithrombic action.

Fractionation of bovine plasma by several methods failed to yield a heparin cofactor preparation of significantly increased potency.

Heparin antithrombic cofactor and normal antithrombin appear to be identical. The major antithrombic action of heparin seems to be related to the acceleration of the interaction between thrombin and normal antithrombin.

RECOMMENDATIONS

Further attempts should be made to isolate a potent cofactor preparation and to dissociate cofactor activity from normal antithrombin, if possible. If these activities can be separated, further quantitative studies should be undertaken on the interactions of the purified fractions with thrombin and heparin.

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OBSERVATIONS ON HEPARIN AND HEPARIN ANTITHROMBIC COFACTOR

I. INTRODUCTION

Heparin has long been known to retard and, in sufficient concentration, to prevent the coagulation of blood (1). Heparin can inhibit the action of thromboplastin on prothrombin (2, 3, 4); it may delay prothrombin conversion by another mechanism mediated through a specific plasma heparin antiprothrombic cofactor (5); it retards the action of thrombin on fibrinogen, especially in the presence of a specific plasma heparin antithrombic cofactor (6); and finally, in high concentrations, heparin is reported to retard fibrin formation by a direct interaction with fibrinogen a reaction distinctly separate from its antithrombic action (7). These functions of heparin may be described as antithromboplastic, antiprothrombic, antithrombic, and antifibrinoplastic activities, according to the site of interference in the blood coagulation process.

There is general agreement that the presence of a factor in blood is required for heparin to exert its powerful antithrombic action. It has been claimed that, in addition to this heparin antithrombic cofactor, there is present in blood a separate normal antithrombin factor which has the ability to inactivate thrombin directly (8, 9).

The present report is concerned with observations on the interactions of heparin, purified thrombin, and purified fibrinogen in the presence and absence of plasma or serum, and with efforts to isolate and characterize the heparin antithrombic cofactor*.

II. EXPERIMENTAL

A. Materials

Fibrinogen was prepared from fresh oxalated bovine plasma by the freezing-thawing technique (10). All preparations used in this

*The term "heparin antithrombic cofactor" is intended to distinguish this activity of plasma and of serum from factors enhancing the antiprothrombic and antilipemic effects of heparin. The term "proantithrombin" (1, 6) implies that this cofactor is a precursor of normal antithrombin to which heparin is added as a prosthetic group for the development of anticoagulant activity. This assumption has never been satisfactorily proven.

study contained more than 90 per cent clottable protein (11). Stock fibrinogen solutions in concentrations of 20 to 30 mg of protein per ml in 0.9 per cent NaCl were stored in small test tubes at -20° C. These were thawed at 37° C and diluted with 0.9 per cent NaCl to a fibrinogen concentration of 5 mg per ml just prior to use.

Bovine thrombin* containing 26 to 27 NIH units of thrombin per mg of salt-free protein was diluted to a concentration of 1000 NIH units per ml in 0.9 per cent NaCl and stored at -20° C. Small aliquots were warmed to 37° C and further diluted in saline to 100 NIH units per ml just prior to use. A limited amount of a highly purified bovine thrombin preparation** containing 500 NIH units of activity per mg was also employed.

Aqueous heparin from several sources*** was diluted to a concentration of 10 NIH**** units per ml in 0.9 per cent NaCl and stored at 4° C.

The reactants were dissolved or diluted either with 0.154 M Krebs buffer of pH 7.8, or with 0.154 M NaCl (0.9 per cent) of pH 7.0 $\pm$ 0.2 in the measurement of the clotting time, carried out at 27° C.

Separation of antithrombic cofactor: Fresh oxalated bovine plasma, defibrinogenated plasma prepared by stepwise addition of 2 to 3 NIH units of thrombin per ml of plasma, or bovine serum obtained either by collecting blood without anticoagulant or by recalcification of oxalated plasma were used as starting material for the fractionation of the heparin cofactor. The following procedures were employed in the fractionation experiments: (a) Ammonium sulfate and isoelectric precipitation; (b) ethanol fractionation: Bovine plasma was diluted ten times with distilled water, the pH adjusted to 6.2 with 0.1 N acetic acid, and the soluble proteins fractionated with cold ethanol at 0° C according to the directions of Cohn and his associates (12); (c) convection electrophoresis of bovine serum in the presence of heparin or of dextran sulfate: Fresh bovine serum was treated with 1 mg of heparin or of

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*Thrombin Topical, Parke, Davis and Co.

**Kindly provided by Dr. W. Seegers, Wayne University College of Medicine, Detroit, Michigan

***Abbott Laboratories, Pure Organics, Inc.; Eli Lilly and Co.; Lederle Laboratories.

****One NIH heparin unit is equivalent to 0.01 mg of crystalline heparin.

dextran sulfate per ml, equilibrated against phosphate buffer of pH 7.4 and u = 0.1 and recycled three times at 4° C in the convection electrophoresis apparatus*, and (d) adsorption on a sulfonic acid resin column.

B. Assay Procedures

1. Heparin Antithrombic Cofactor - A series of 10x75 mm test tubes was arranged as shown in Table 1. The concentration of the individual components in the different combinations was varied by dilution of the stock reagent. The final volume of the clotting mixture was always 2.0 ml. Each determination was performed in duplicate or triplicate, and the average value used in the calculations of the clotting times.

Variation of thrombin concentration in mixture A provided a thrombin assay curve as a standard reference for each series of experiments. Results in column B, compared with those in A, indicated the intrinsic antithrombic effect of the cofactor preparation. Similarly, results in column C provided a measure of the intrinsic antithrombic action of heparin. Differences between D and C were attributed to heparin cofactor activity, provided that the cofactor preparation showed little or no effect on the clotting time in the absence of heparin, column B.

The components of the clotting mixture were added to the test tube in the order listed in Table 1, the clotting time measurement proceeding from the moment of the introduction of thrombin. Incubation of thrombin and heparin together at 27° C, or variable periods of incubation of the remaining three components with one another prior to the constitution of the complete clotting mixture did not alter the results in the control experiments.

2. Normal Antithrombin - Aliquots (1 ml) of serum, of defibrinogenated plasma, or of test fractions, were mixed with 500 or 1000 NIH units of thrombin and diluted to a final volume of 2.0 ml with 0.9 per cent NaCl. The solutions were incubated at 27° C and tested for residual thrombic activity at intervals up to 60 min by transferring 0.1 ml of each mixture to a 10x75 mm test tube containing 1.5 ml of 0.9 per cent NaCl and 0.4 ml of 0.5 per cent fibrinogen. Loss of thrombic activity was determined by comparison with control solutions of 500 or 1000 units of thrombin in 2.0 ml of 0.9 per cent NaCl tested at the same time, as well as by reference to a standard thrombin assay curve.

*Beckman Instruments, Inc.

3. Clotting Time Determination - Thrombin was rapidly discharged into the fibrinogen solution and a glass rod 5 mm in diameter, spun at 1000 rpm by a small electric motor, was immediately inserted to the full depth of the clotting mixture. The clear solution became opalescent 1 to 5 sec prior to the actual clot formation, but only the first appearance of definite fibrin strands was taken as the clotting-time end point. Incubation of reactants and clotting time determinations were carried out at 27° C unless otherwise specified.

III. RESULTS

In order to establish conditions for a valid quantitative assay of heparin antithrombic cofactor activity, the concentration of each component in the test system was studied as an independent variable. The clotting time of a mixture of thrombin and fibrinogen within the range studied was found to be inversely related to the concentration of each component (Figs. 1 and 2). The determination of the clotting time was less precise when the clotting time exceeded 60 sec in a 1 mg per ml fibrinogen solution and also when the fibrinogen concentration was less than 0.5 mg per ml; there was usually an error of several seconds. Similarly, when the clotting time was less than 10 sec, relatively large variations in thrombin concentration were required to produce an appreciable change in clotting time.

On the basis of the data presented in Figures 1 and 2, a reaction mixture containing 5 NIH units of thrombin per ml and 1 mg of fibrinogen per ml was selected as the standard against which to test the action of heparin and its cofactor. The standard reaction mixture gave a clotting time of approximately 13 sec in 0.9 per cent NaCl (pH 7.0) at 27° C.

Using several different preparations of heparin, fibrinogen, and thrombin (including a highly purified thrombin preparation of Dr. W. Seegers), it was uniformly noted that low concentrations of heparin produced a small but distinct prolongation of clotting time in a mixture of purified thrombin and purified fibrinogen (Fig. 3, Table 2). This prolongation was dependent upon the concentration of thrombin and heparin (Fig. 4) and was not appreciably affected by changes in fibrinogen concentration. The slightly greater effect of heparin observed in diluted fibrinogen solutions, less than 1 mg per ml, (Fig. 5) was probably an artifact resulting from the difficulty in precisely recognizing the end point of clotting time in dilute fibrinogen solutions.

Comparison of the heparin clotting time with a simultaneously measured standard thrombin assay (Fig. 3) clearly indicated that a

constant fraction of the thrombin present was inactivated by any given concentration of heparin. Within the limits selected for optimal sensitivity and precision of clotting time measurements (CT = 10 to 60 sec thrombin = 0.6 to 10 NIH units per ml, fibrinogen = 1.0 mg per ml), a maximal clot retarding action of heparin was attained at 1 to 2 NIH units of heparin per ml. This corresponded to a reduction of approximately 60 per cent in effective thrombin concentration (Fig. 6). A thrombin concentration in excess of 20 NIH units per ml was enough, therefore, to completely obscure the action of as many as 50 NIH units of heparin per ml, because of the asymptotic nature of the thrombin assay curve at high concentrations of thrombin and of the limitation in the action of heparin alone upon thrombin.

The addition of plasma, defibrinogenated plasma or serum often unpredictably shortened the clotting time in the absence of heparin. This was presumed to be due either to a non-specific stabilizing action of added proteins (13) or to a "specific" accelerating action of a factor present in the albumin fraction (14). This effect was markedly reduced, or eliminated entirely, by a 3 to 5-fold dilution of the plasma or serum with 0.9 per cent saline.

The antithrombic effect of heparin was remarkably enhanced in the presence of the heparin antithrombic cofactor (serum) and under these conditions the instantaneous reduction in effective thrombin concentration was proportional to the amount of cofactor (serum) added (Fig. 7). Reduction of the heparin concentration to less than 0.1 NIH units per ml led to a marked diminution or total loss of the characteristic antithrombic activity of the heparin plus cofactor combination (Table 2, a). On the other hand, increases in heparin concentration above 1 to 2 NIH heparin units per ml (maximal effect in the absence of the cofactor) did not significantly increase the antithrombic potency of the cofactor at the levels tested (Table 2, b).

Dilution of the cofactor solution (serum) led to an apparent increase in its specific activity (i.e., units of thrombin inactivated per mg of cofactor), while increasing dilution of thrombin led to an apparent diminution in specific activity of the cofactor (Table 3). Increasing the concentration of fibrinogen above the standard level of 1.0 mg per ml did not affect the results.

The sequence of addition of reactants, as noted earlier, had no effect on the clotting time of the thrombin-fibrinogen or the thrombin-heparin-fibrinogen systems. This no longer applied in the experiments with the cofactor, however, for admixture of cofactor with thrombin

prior to the addition of fibrinogen led to a loss of thrombin, and also to a loss of heparin cofactor activity. This difference was more marked if (a) high concentrations of thrombin were used in setting up the stock solutions for the tests, or when (b) the thrombin was allowed to react with the cofactor preparation for prolonged periods of time before testing.

These observations indicated the presence of normal antithrombic activity, and since there is general agreement on the course of the thrombin-normal antithrombin interaction (7, 8, 15), it was not investigated in detail. However, at relatively high concentrations of thrombin and serum the normal antithrombin inactivated a major fraction of the thrombin within 30 to 60 sec. Thereafter, residual thrombin was inactivated at a progressively slower rate (Fig. 8). As normal antithrombin became depleted in this reaction heparin cofactor activity was reduced in a similar manner, i.e., an initial rapid loss of cofactor activity was followed by a gradual progressive loss thereafter. Heating of defibrinogenated plasma or of serum at 50° C to 65° C for 30 to 60 min led to roughly parallel decreases in both normal antithrombin and heparin cofactor activities as well (Fig. 9). This finding is in good agreement with similar observations of other investigators (6, 9).

The complex interrelationships of these factors required a rigidly standardized assay method for interpretable results in assaying the heparin cofactor activity of partially purified fractions from plasma. The schema outlined in Table 1 column D was employed, using 100 NIH units of thrombin per ml, 10 NIH units of heparin per ml, and a 1.0 per cent solution of the cofactor fraction. Clotting times all fell within the range of 10 to 60 sec.

Upon fractionation of bovine plasma with ammonium sulfate, heparin cofactor activity was found to be in the fraction precipitating between 50 to 70 per cent saturation. The crude globulin fraction obtained between 30 to 50 per cent saturation usually exhibited increased cofactor activity as well, but the active material shifted into the 50 to 70 per cent ammonium sulfate fraction on a single refractionation. Subfractionation of the 0.5-0.7 SAS precipitate did not result in any increase in specific activity and was generally associated with a loss of activity. As outlined in a representative experiment (Table 4), the active fractions obtained by ammonium sulfate precipitation exhibited only a modest increase in specific activity compared with the starting material.

Heparin cofactor activity was found to be concentrated in the fraction precipitating within the limits of 15 to 25 per cent ethanol (v/v). The

specific activity of this fraction, however, was no greater than that obtained on ammonium sulfate fractionation.

Heparin combines with certain plasma proteins to form reversible complexes with an electrophoretic mobility which is intermediate between free heparin and free protein (16, 17, 18). It was found (16, 17, 18) that heparin forms complexes with components of α_2- and β-globulins, corresponding in general with the solubility characteristics of the active cofactor fractions obtained in the ammonium sulfate and ethanol fractionation procedures. In the present study, no detectable increase in specific cofactor activity was found for the fast fraction in the convection electrophoresis.

Passage of bovine serum through a sulfonic acid resin column washed in distilled water did not remove any detectable amount of cofactor from the starting material.

IV. DISCUSSION

According to current theory heparin becomes an active antithrombic agent only after combining with a specific plasma protein, the heparin antithrombic cofactor. This concept stems from the claims that heparin is completely devoid of anticoagulant action upon purified thrombin and fibrinogen in the absence of another factor (1, 6). This theory has been modified to state that the intervention of a cofactor is required only below a critical concentration of heparin (8). In support of this view, the anticoagulant activity of a high concentration of heparin upon purified thrombin and fibrinogen systems has been attributed to a direct effect of heparin on fibrinogen rather than on thrombin (19).

The repeated observations recorded here of a distinct antithrombic activity of low concentrations of heparin in the absence of added cofactor might at first be attributed to cofactor impurities in the thrombin, heparin, or fibrinogen preparations used. This explanation appears improbable, however, in view of the observations that the direct antithrombic action of heparin was identical for every heparin preparation, irrespective of source, for a purified thrombin (550 NIH units per mg) far more potent than the preparations used in the early studies (1, 6), and for every batch of purified fibrinogen. Since the completion of this work there have been two other reports of an antithrombic action of heparin in purified systems in the absence of added cofactor (15, 20).

Examination of the data purporting to demonstrate a complete lack of intrinsic antithrombic activity of heparin (1, 6, 8) reveals that heparin

alone did, in fact, prolong the clotting time of purified thrombin and fibrinogen although the delay expressed in seconds was "negligible" by comparison with that produced by the combination of heparin and cofactor. In these experiments, furthermore, the initial concentration of active thrombin was high enough (about 10 NIH units per ml) to produce a clotting time of 10 to 12 sec or less. These conditions obscured the inactivation of a major fraction of the thrombin present and thereby masked the a tion of heparin for reasons outlined earlier.

The heparin-thrombin reference control (Table 1, column C) was found to be preferable to the standard thrombin control (Table 1, column A) in analyzing the action of the heparin cofactor, for in this way the observed maximal inhibition was related to the addition of a single factor at a time. The fundamental difference in the action of heparin (inactivating a constant fraction of active thrombin) and of cofactor (inactivating a constant amount of active thrombin) was revealed only by such analysis.

The demonstration of an inhibitory action of heparin upon purified thrombin and fibrinogen which is dependent upon the concentration of thrombin and heparin, but not upon fibrinogen concentration, leads to the conclusion that heparin reacts directly and reversibly with thrombin itself. Complexes of heparin with many proteins are known (17, 18), and a thrombin-heparin complex has been demonstrated electrophoretically as well (20). The development of an essentially irreversible immediate inactivation of thrombin by the addition of heparin cofactor to a thrombin-heparin mixture suggests the following general sequence of reactions:

a Normal antithrombin

$$T_a \rightleftharpoons T_i \xrightarrow{C} T_iC$$

b. Heparin and cofactor

$$T_a + H \rightleftharpoons TH \rightleftharpoons T_i + H \xrightarrow{C} T_iC$$

where T_a = active thrombin; T_i = inactive thrombin; H = free heparin; TH = intermediate thrombin-heparin complex; C = cofactor and/or normal antithrombin; T_iC = thrombin-antithrombin complex.

This hypothetical formulation is equivalent in effect to the thesis that heparin accelerates the reaction between thrombin and normal antithrombin (19). The close relationship, or identity, of heparin cofactor and normal antithrombin is supported by the parallel response of these

activities on salt fractionation and heat inactivation (6), by persistent association of the two activities in the most potent preparation of cofactor described to date (21), and by the decline of cofactor activity upon depletion of normal antithrombin of plasma or serum by thrombin shown here and also in another publication (20). The recent studies of Waugh and Fitzgerald (22) comparing the effects of heparin added to plasma with those obtained on an isolated heparin cofactor preparation also suggest that an independent heparin cofactor does not exist in plasma. These investigators suppose that heparin accelerates the rate at which antithrombin inactivates thrombin. According to Burstein and Guimand (23), heparin increases the affinity of antithrombin for thrombin.

The data presented here as well as the observations of others, cited, are in harmony with the concept of a close relationship, or identity, of heparin antithrombic cofactor and normal antithrombin. Definite proof of the identity of heparin cofactor and normal antithrombin must await a more detailed study of the kinetics of both antithrombic systems under proper test conditions and the demonstration of a constant ratio of these activities, under valid test conditions, for highly purified preparations.

V. SUMMARY

The antithrombic activity of heparin and of bovine plasma and serum has been studied. Low concentrations of heparin, in the absence of the heparin cofactor, exhibit a moderate, reversible antithrombic action. The maximal effect of heparin in the absence of the cofactor is equivalent to inactivation of approximately 60 per cent of the thrombin present. The addition of cofactor to a mixture of heparin and thrombin leads to a further immediate and irreversible inactivation of part or all of the thrombin present, in proportion to the amount of cofactor added.

The interpretation of the observed data is in agreement with the assumption that heparin catalyzes a reversible reaction between an active and an inactive form of thrombin and that the latter is bound to the heparin cofactor or normal antithrombin without further intervention of heparin. The action of heparin therefore becomes, in effect, an acceleration of the reaction between thrombin and normal antithrombin.

VI. RECOMMENDATIONS

Further fractionation experiments should be carried out in an attempt to separate heparin antithrombic cofactor from normal antithrombin activity, if possible, and to obtain preparations of high potency. Quantitative measurements of the kinetics of the interaction between thrombin

and heparin, heparin cofactor, and normal antithrombin should further clarify the problem.

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TABLE 1

SCHEMA FOR ASSAY OF HEPARIN ANTITHROMBIC COFACTOR

	A	B	C	D
1. Buffer or Saline (0.15M)	1.5*	1.4	1.4	1.3
2. Fibrinogen (0.5%)**	0.4	0.4	0.4	0.4
3. Cofactor (0.01-0.1%)**	-	0.1	-	0.1
4. Heparin (1-1000 NIH u/ml)**	-	-	0.1	0.1
5. Thrombin (25-1000 NIH u/ml)**	0.1	0.1	0.1	0.1
	2.0	2.0	2.0	2.0

*Volume of reactant in ml.

**Range of concentration expressed in gm per 100 ml or units of activity per ml.

TABLE 2

EFFECT OF HEPARIN AND COFACTOR ON CLOTTING TIME

a.	THROMBIN u/ml	FIB. mg/ml	HEPARIN u/ml	COFACT. cc/ml	C.T. sec.	b.	THROMBIN u/ml	FIB. mg/ml	HEPARIN u/ml	COFACT. cc/ml	C.T. sec.
	2.0	1.0	0	0	24.2		5.0	1.0	0	0	13.6
	2.0	1.0	1.0	0	38.5		5.0	1.0	0.25	0	15.8
	2.0	1.0	0.1	0	29.7		5.0	1.0	0.25	0.05	>300
	2.0	1.0	1.0	0.05	>600		5.0	1.0	0.25	0.013	26.5
	2.0	1.0	0.1	0.05	28.2		5.0	1.0	0.50	0.013	33.5
							5.0	1.0	2.50	0.013	45.0
							5.0	1.0	5.00	0.013	47.4
							5.0	1.0	10.0	0.013	49.0
							5.0	1.0	25.0	0.013	42.3

TABLE 3

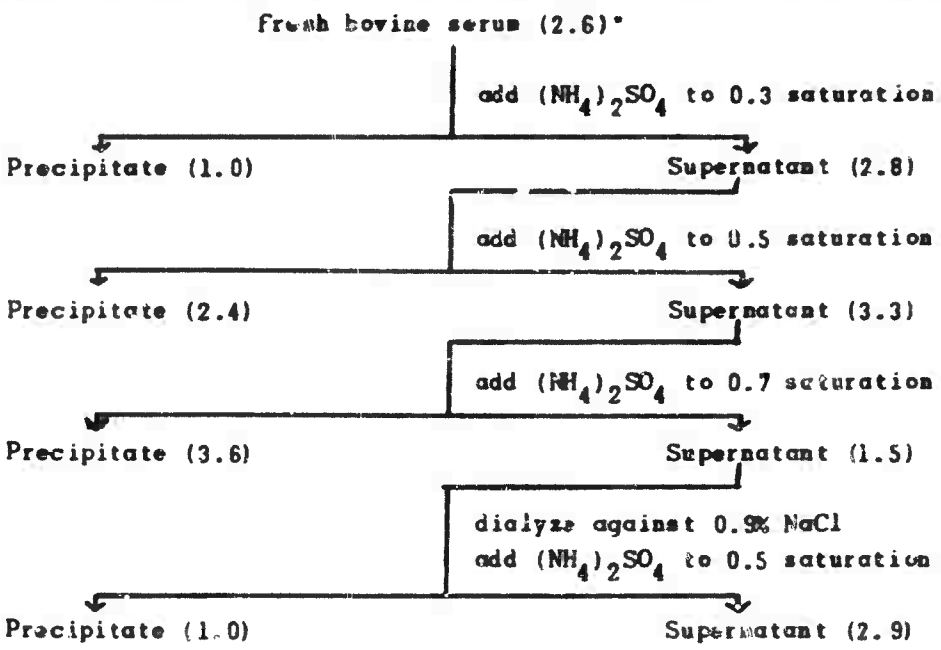
EFFECT OF THROMBIN AND SERUM CONCENTRATION ON APPARENT COFACTOR ACTIVITY

Thrombin NIH u/ml	Fibrinogen cy/ml	Heparin NIH u/ml	Serum cc/ml	Clotting Time seconds	Cofactor Activity* units/ml
5.0	1.0	5	0	18.4	-
2.5	1.0	5	0	25.9	-
1.25	1.0	5	0	38.7	-
0 63	1.0	5	0	59.4	-
5.0	1.0	5	0.0500	50.1	168
5.0	1.0	5	0.0250	42.3	237
5.0	1.0	5	0.0170	36.4	290
5.0	1.0	5	0.0125	34.4	347
2.5	1.0	5	0.0500	>180	-
2.5	1.0	5	0.0250	>180	-
2.5	1.0	5	0.0170	65.0	250
2.5	1.0	5	0.0125	54.5	295

*One unit of cofactor activity is equivalent to inactivation of one NIH unit of thrombin in the presence of heparin (see Fig. 7). The apparent cofactor activity of serum noted in the table is calculated by dividing the number of units of thrombin inactivated by the cofactor by the concentration of serum in cc per ml of the clotting mixture.

TABLE 4

AMMONIUM SULFATE FRACTIONATION OF BOVINE SERUM FOR HEPARIN COFACTOR



*Specific activity of heparin antithrombic cofactor in units of thrombin inactivated per mg of protein in standard test procedure.

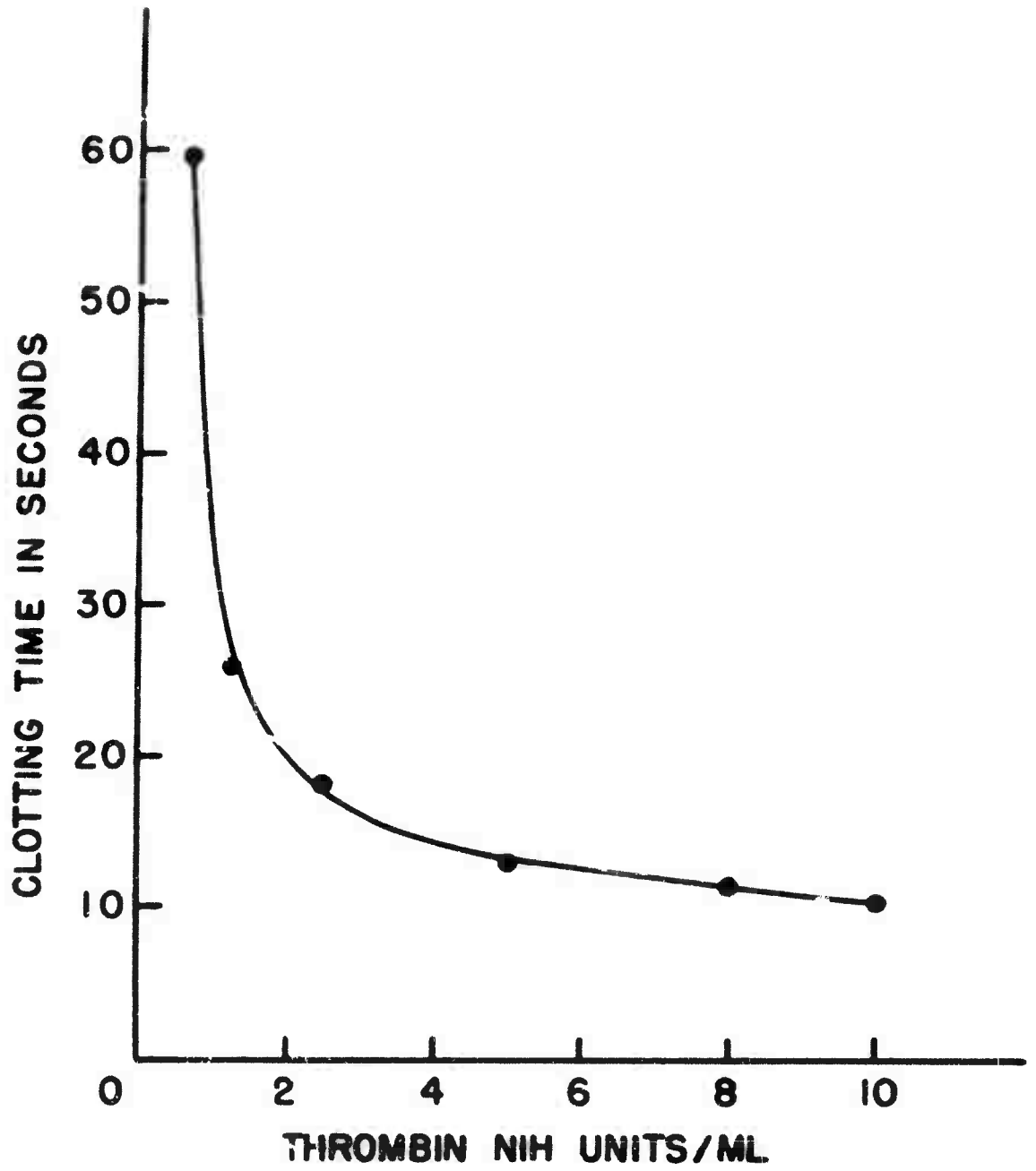


Fig. 1. Thrombin assay curve at 27° C in 0.154 M NaCl. Under these conditions thrombin is approximately 1/2 as active as in the NIH standard procedure.

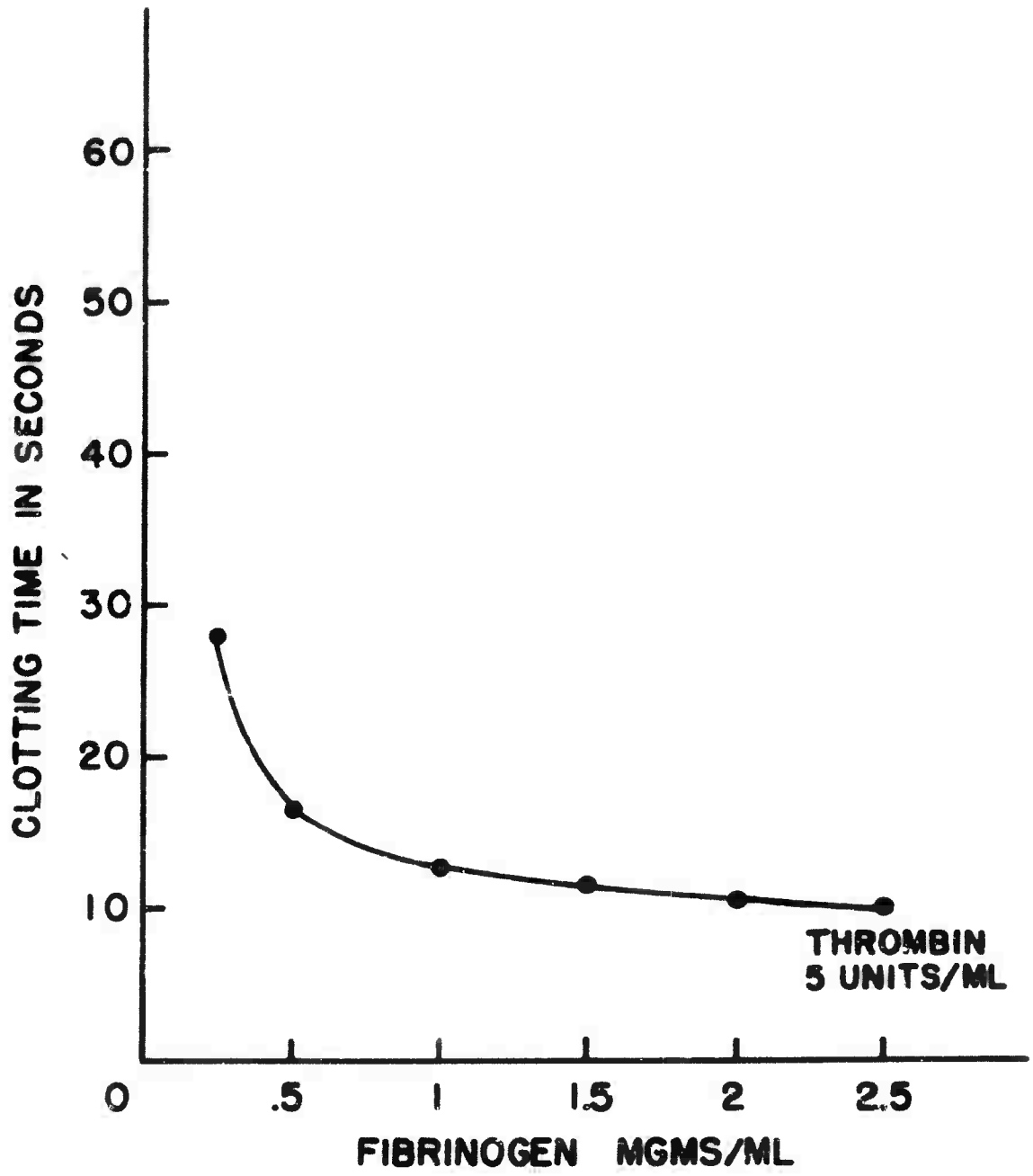


Fig. 2. Effect of fibrinogen concentration on clotting time induced by final concentration of 5 NIH units of thrombin per ml. Clots formed in 0.25 and 0.50 mg/ml fibrinogen solution were thin and difficult to detect.

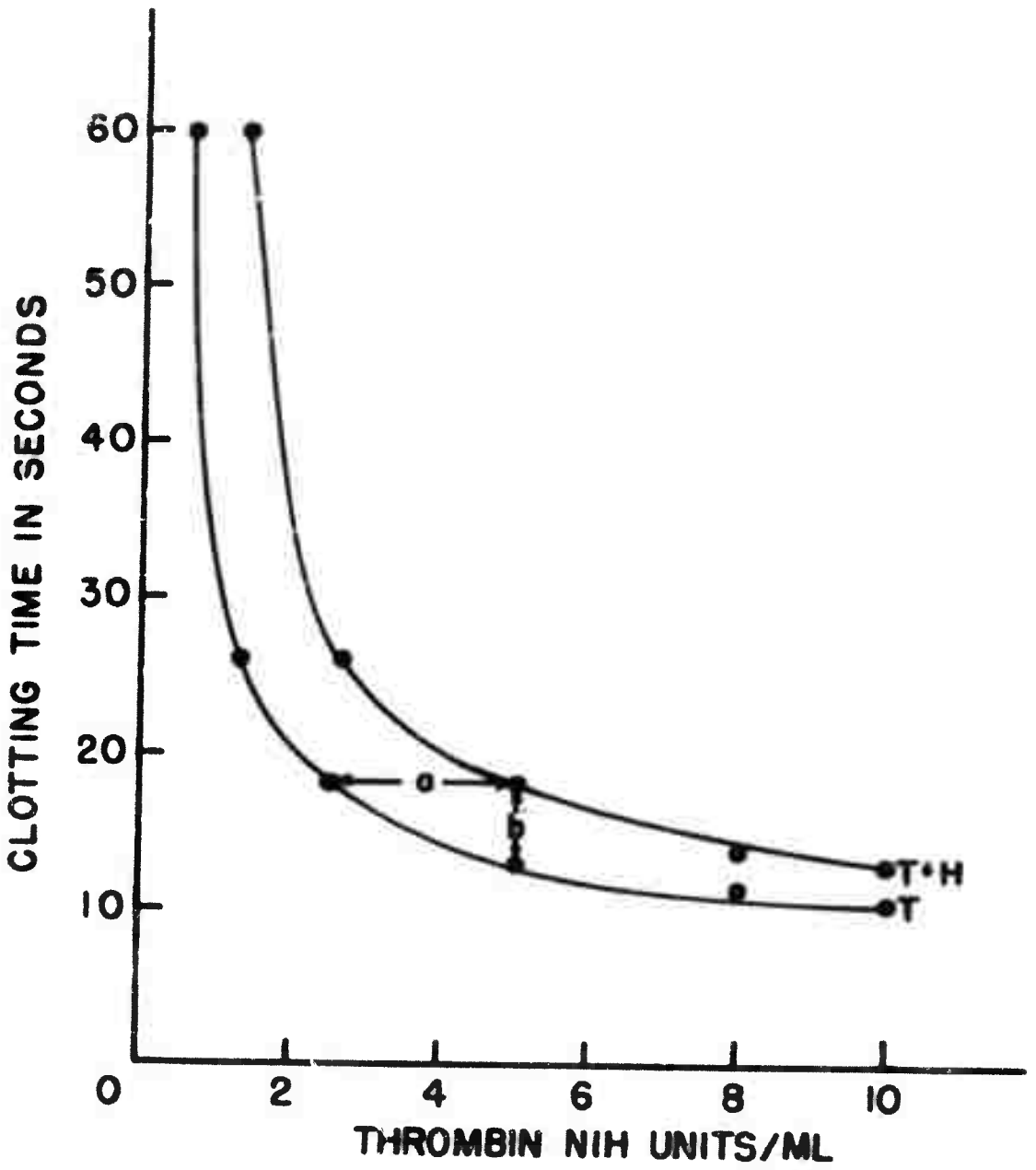


Fig. 3. Effect of heparin on the thrombin-fibrinogen reaction. Lower curve (T) is standard thrombin assay in method. Upper curve (T + H) is parallel determination in the presence of 0.5 NIH heparin units per ml. The displacement of (T + H) along the abscissa, "a" is a measure of units of thrombin inactivated. Displacement of (T + H) along the ordinate is the prolongation of clotting due to heparin, and becomes smaller and smaller as thrombin concentration is increased.

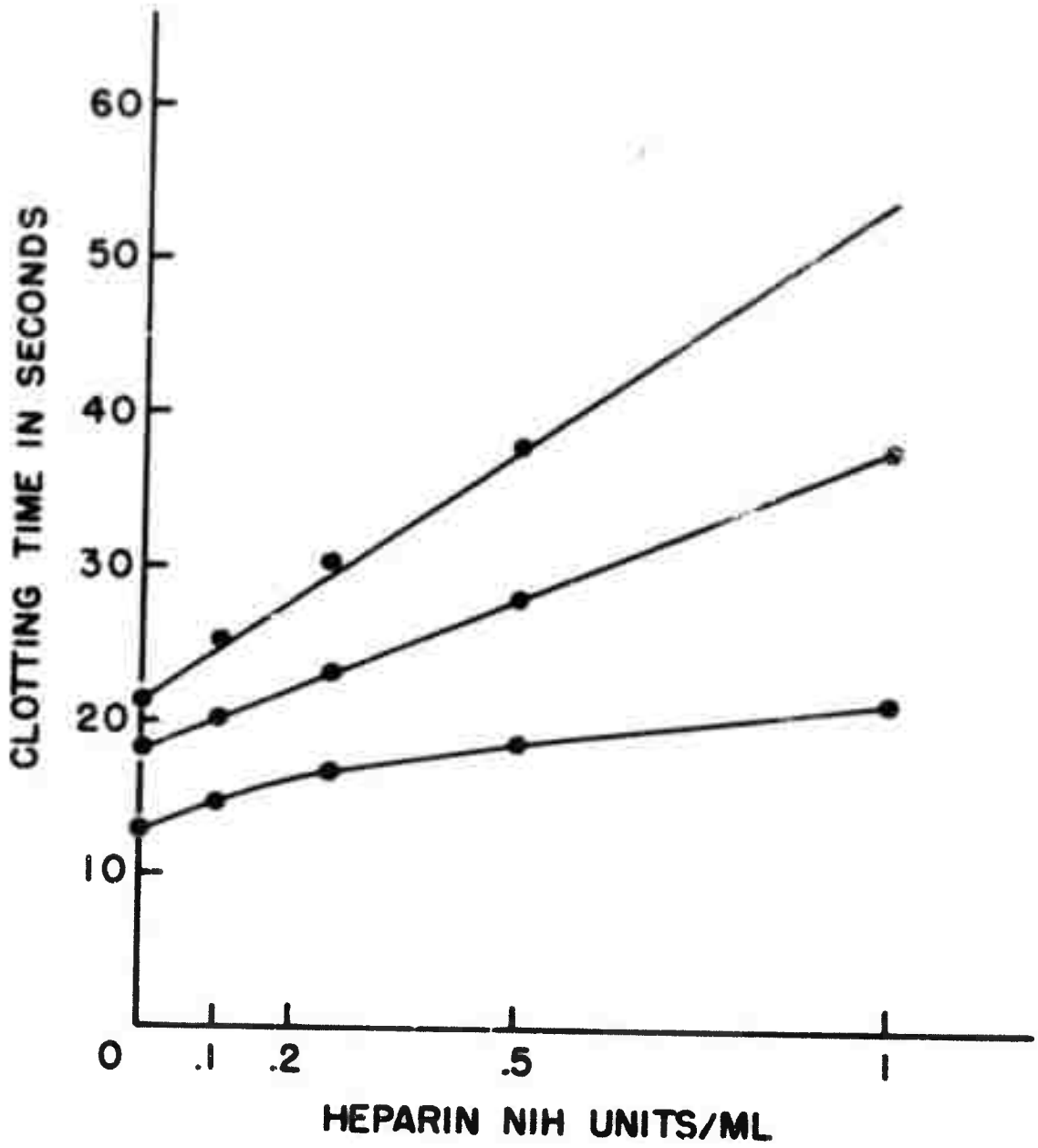


Fig. 4. Effect of heparin concentration on clotting time. Upper curve determined in presence of 2 units of thrombin per ml, middle curve with 2.5 units of thrombin per ml, and lower curve with 5.0 units of thrombin per ml.

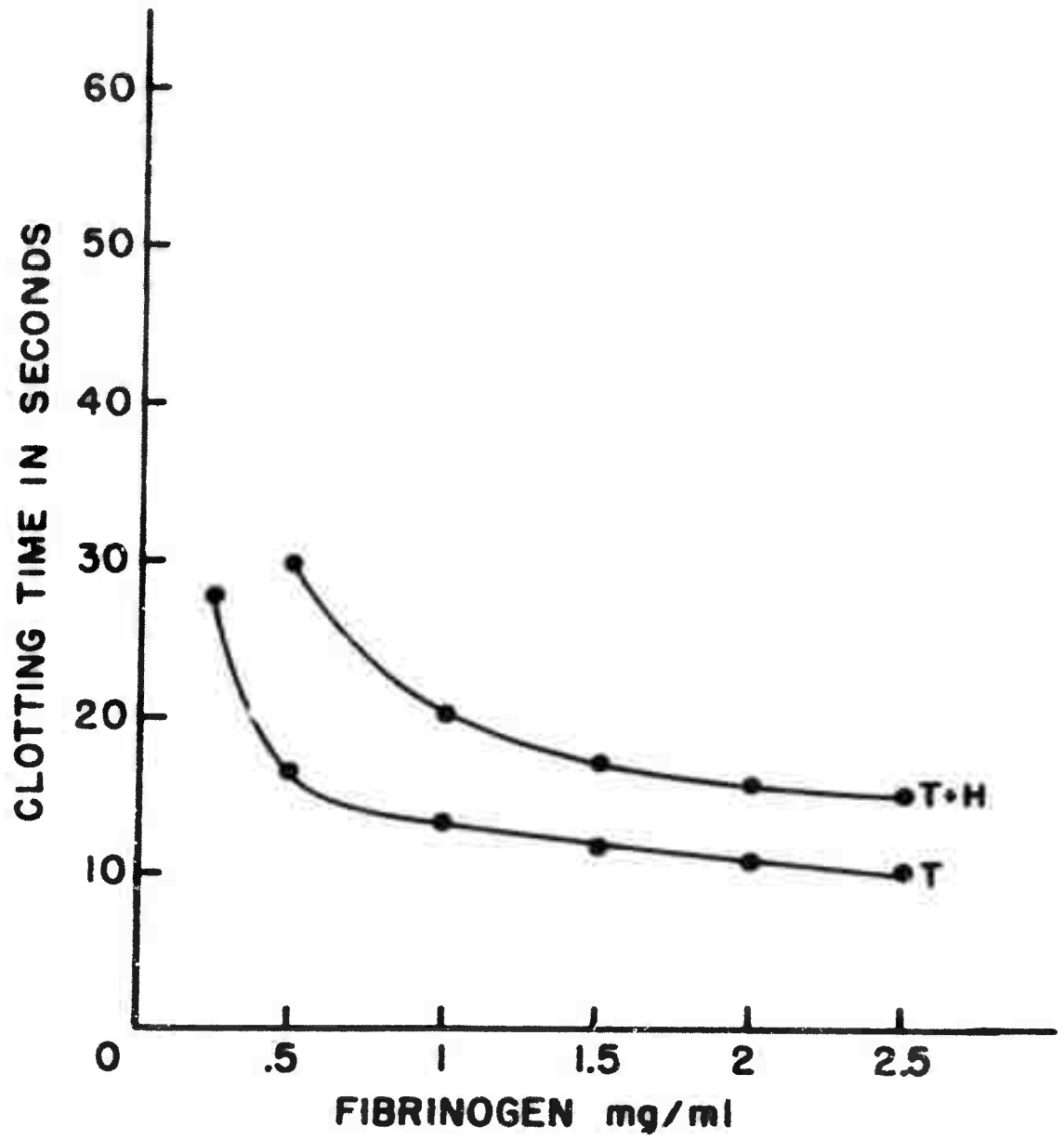


Fig. 5. Effect of fibrinogen concentration on the heparin-thrombin clotting time. Upper curve (T + H) obtained with 5 NIH units of thrombin and 0.5 NIH units of heparin per ml. Lower curve (T) obtained with 5 NIH units of thrombin per ml. Note that upward displacement of (T+H) curve from (T) is not overcome by increasing fibrinogen concentration from 1.0 to 2.5 mgm per ml. Clots obtained with (T + H) in 0.5 mgm per ml fibrinogen were very hard to time accurately.

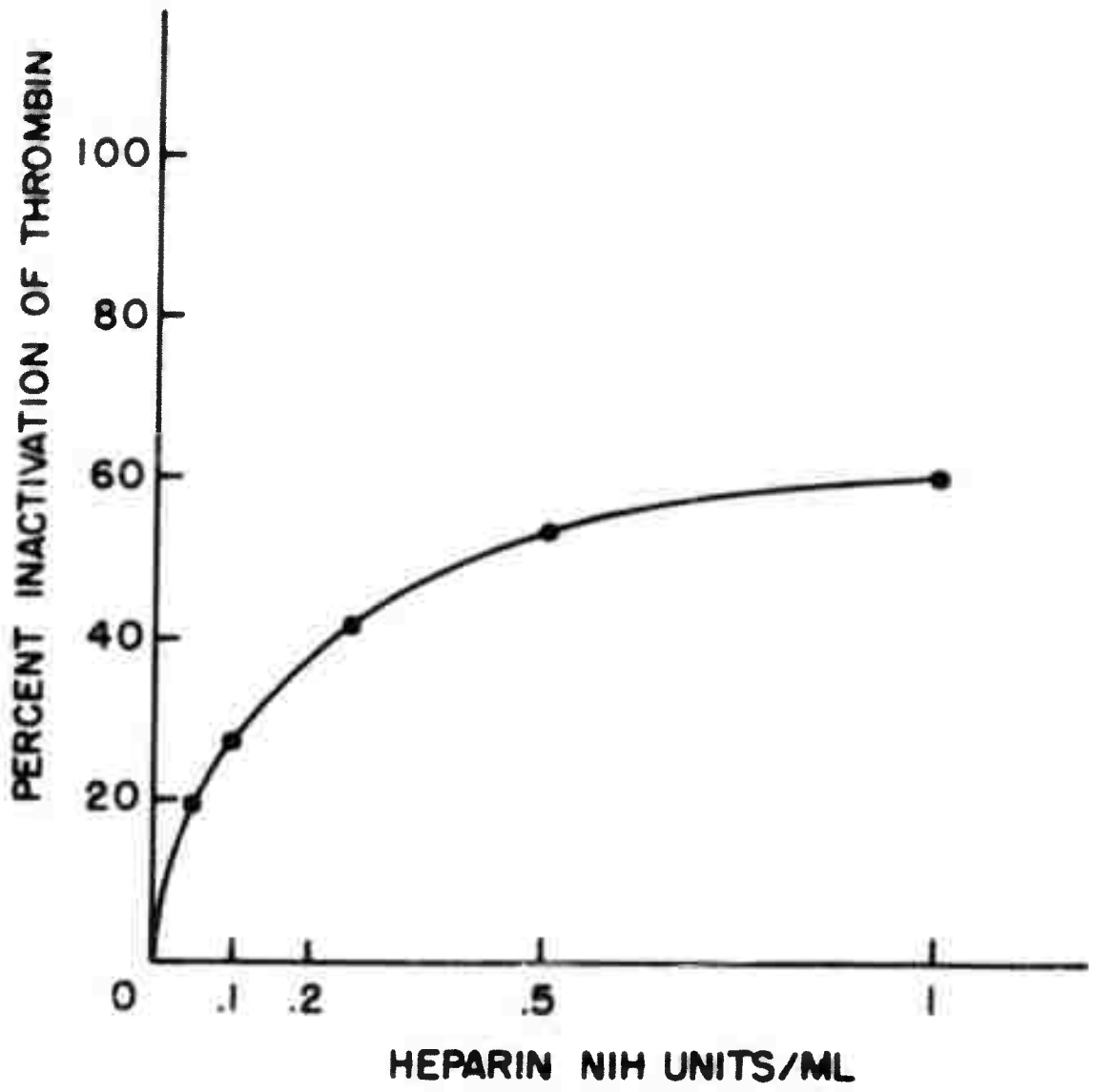


Fig. 5. Effect of heparin concentration on degree of thrombin inactivation. The clotting times of heparin-thrombin mixtures are converted to equivalent units of active thrombin by projection onto a standard thrombin assay done in parallel (Fig. 3).

$100 = \frac{\text{T equivalent}}{\text{T added}}$ x 100 equals per cent thrombin inactivated.

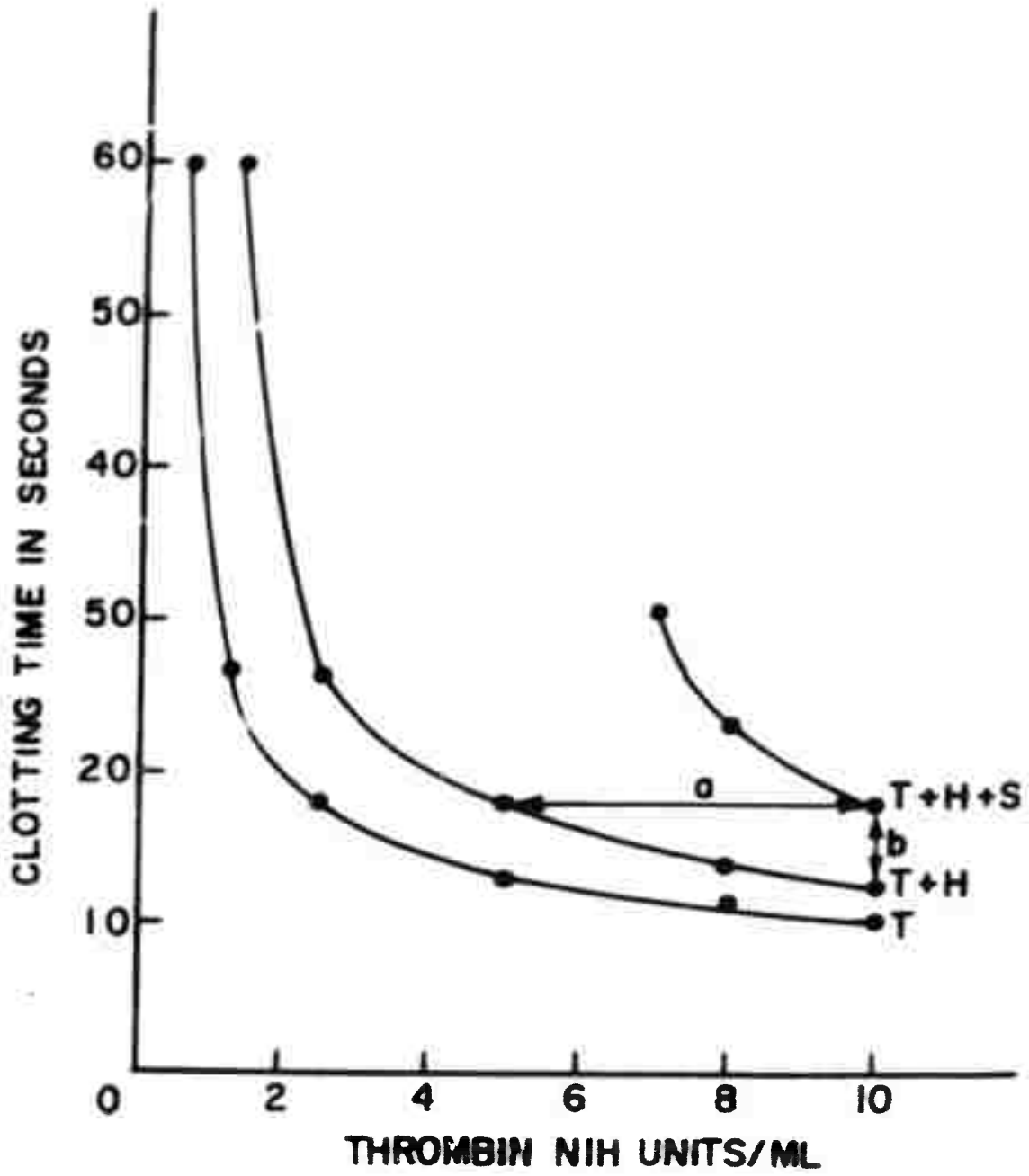


Fig. 7. Effect of bovine serum (cofactor source on the thrombin-heparin-clotting time. Upper curve (T + H + S) obtained with 0.5 NIH units of heparin and 0.025 ml serum per ml reaction mixture. Middle curve (T + H) obtained with 0.5 NIH units of heparin. Lower curve is standard thrombin assay as described. The displacement of (T + H + S) from (T + H) is used for calculation of effect of serum, and does not vary with thrombin concentration in direction of the horizontal axis within range of limits of reasonable precise clotting time measurements.

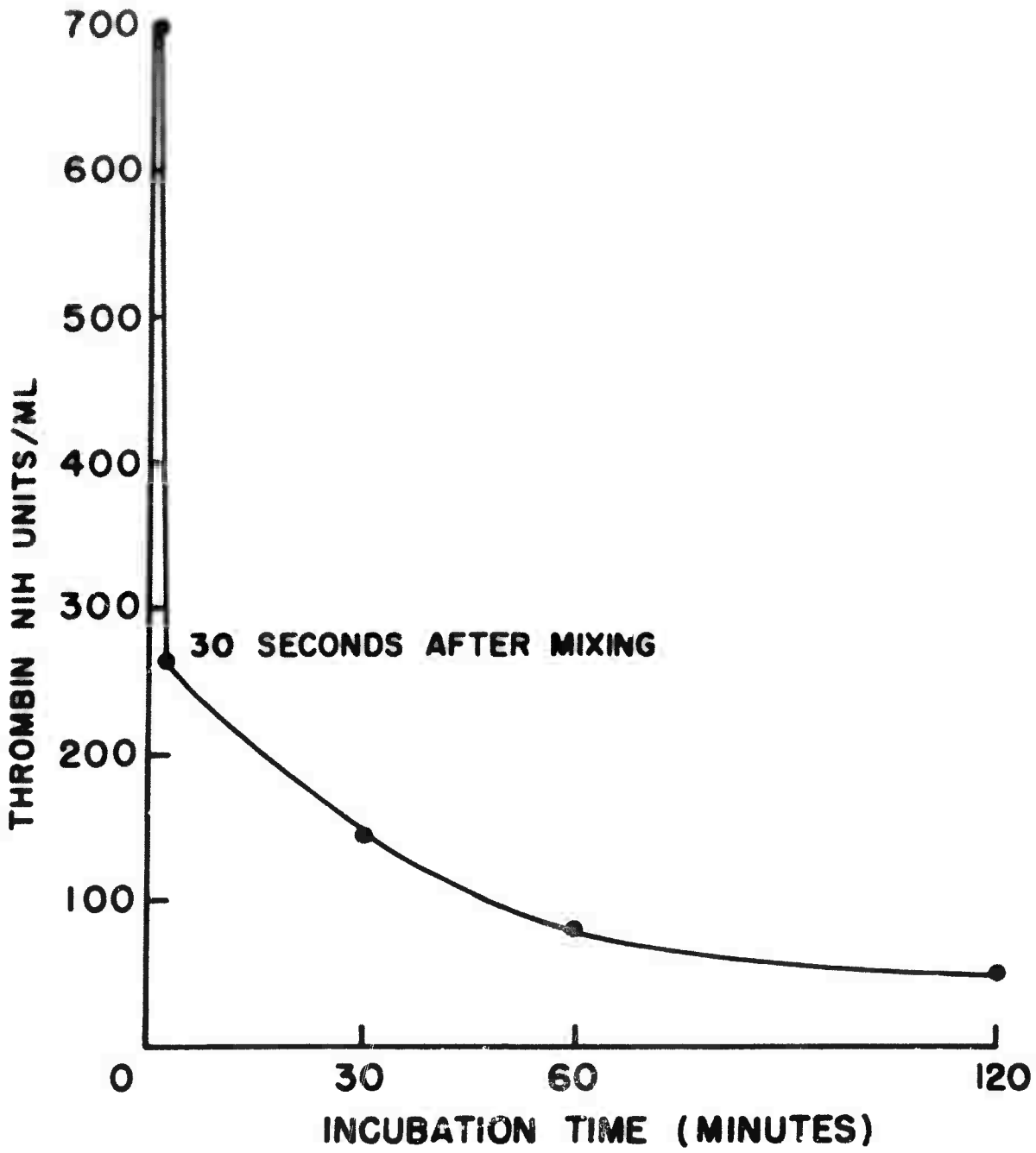


Fig. 8. Normal antithrombin reaction of bovine serum. One ml of a concentrated thrombin solution (1400 NIH units/ml) is mixed with 1 ml of bovine serum and residual thrombin measured by transferring small aliquots (0.01 ml without dilution) to a standard fibrinogen mixture. Incubated at 27° C.

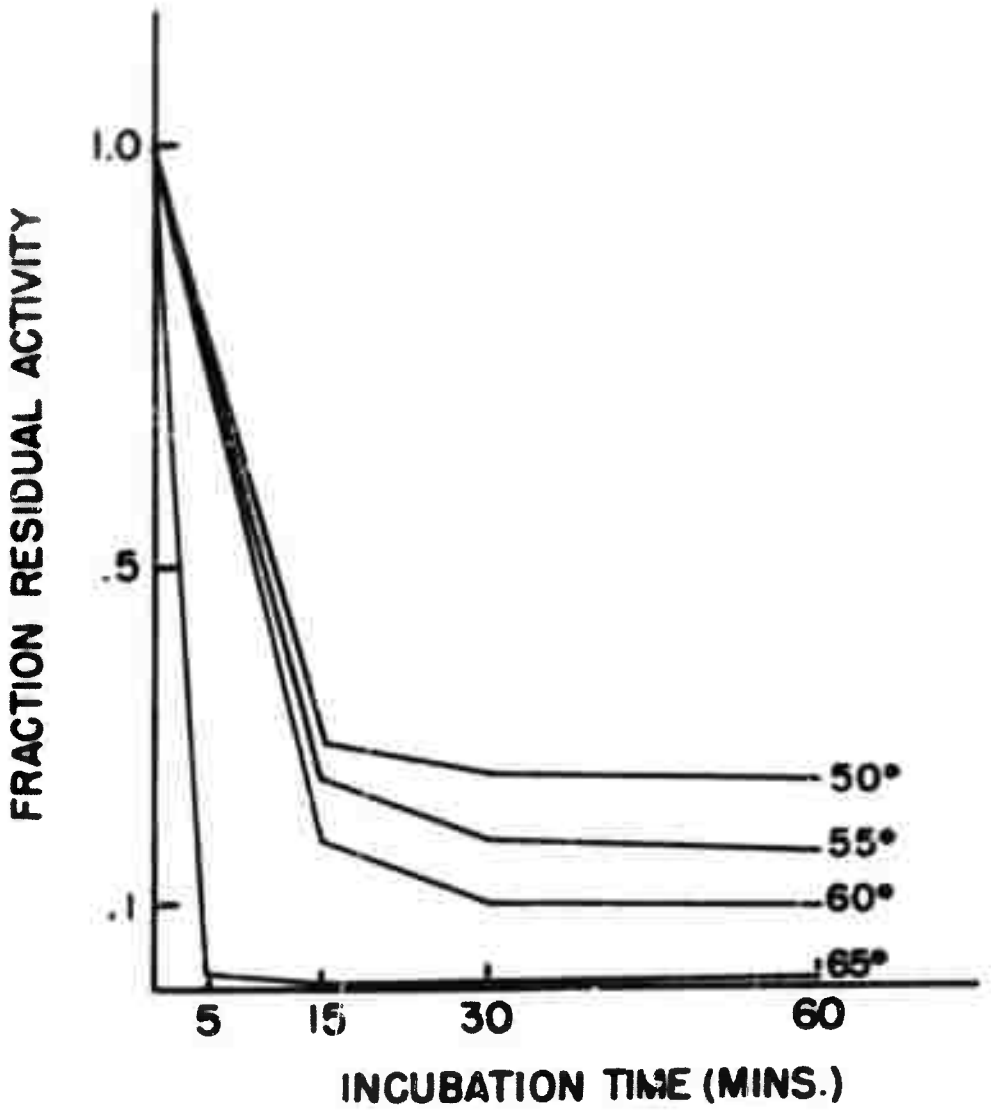


Fig. 9. Thermal inactivation of heparin cofactor. Bovine serum was incubated at the indicated temperatures for variable periods of time, then rapidly chilled and tested for cofactor in the standard assay. Normal antithrombin activity diminished in a similar manner, but quantitative assay was not attempted.

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